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Cytochrome c mediates apoptosis in hypertensive nephrosclerosis in Dahl/Rapp rats

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Background. Renal damage from hypertension is the second most common cause of end-stage renal failure in the United States. The pathogenesis of this process is incompletely understood. The Dahl/Rapp salt-sensitive (S) rat is a model of low-renin hypertension, but these rats also develop renal lesions that are virtually identical to human hypertensive nephrosclerosis.

Methods. To explore apoptosis as a mechanism of progressive renal injury in S rats, age- and sex-matched S and Sprague-Dawley (SD) rats were placed on either 0.3 or 8.0% NaCl diets, which were continued for 21 days.

Results. At day 7, renal histology appeared relatively normal, but by day 21 on the high-salt diet, S rats displayed morphological evidence of severe renal injury that included glomerulosclerosis, arteriolosclerosis, and tubulointerstitial damage. Apoptosis was demonstrated in kidneys of hypertensive S rats by day 7. Cytoplasmic content of cytochrome c was increased in the kidney cortex of hypertensive S rats, and isolated mitochondria showed inappropriate release of cytochrome c sufficient to activate caspase-3 in vitro. Activation of caspase-9 and caspase-3 was observed only in kidney cortex from hypertensive S rats.

Conclusions. Kidneys from hypertensive S rats display apoptosis related to mitochondrial release of cytochrome c and activation of caspase-9 and caspase-3. The findings support a primary role of cytochrome c release and apoptosis in the pathogenesis of hypertensive nephrosclerosis in S rats.

As many as 43 million individuals in the United States have high blood pressure [1]. The frequency of occurrence of clinically important end-organ kidney damage is about 1 in 2500 hypertensive patients, making hypertensive nephrosclerosis the second most common cause of end-stage renal disease [2]. The problem is enormously significant, but the pathogenesis of hypertensive renal damage remains incompletely defined. An important genetic model of hypertension developed from the pioneering work of Lewis K. Dahl, who produced from the Sprague-Dawley (SD) line a strain of rat that was genetically prone to develop high blood pressure in response to an increase in dietary salt (8.0% NaCl) [3, 4]. The Dahl salt-susceptible strain was completely inbred by John P. Rapp to yield a line that was virtually homozygous at all genetic loci, thus fixing the characteristics of the strain [4]. When started on 8.0% NaCl diet, Dahl/Rapp salt-sensitive (S) rats rapidly and uniformly developed low-renin hypertension and died within weeks from hypertensive nephrosclerosis [5]. The major renal lesions include arteriolosclerosis, glomerulosclerosis, and interstitial scarring with tubular cell dropout; the pathology is virtually identical to human hypertensive nephrosclerosis. Because of the rapid development of renal failure and the reproducibility of the phenotype, the S strain of rat provides a unique means to investigate the pathogenesis of hypertensive nephrosclerosis. In addition, this model may be very useful in understanding hypertensive nephrosclerosis that occurs in human low-renin essential hypertension, especially in defined, more homogeneous populations such as the black population described by Grim et al [6].

A detailed understanding of the molecular mechanisms of apoptosis, or programmed cell death, has promoted interest in this process in disease states in which fibrosis is the outcome. Although apoptosis is a physiological event that controls cell populations, unregulated apoptosis results in glomerular and tubulointerstitial fibrosis in a variety of disease processes [7–9]. There are basically two pathways that promote apoptosis: signals intrinsic to the cell and stimulating signals from the cell surface, such as Fas. The intrinsic signals of apoptosis generally center on mitochondria and particularly cytochrome c, a nuclear-encoded enzyme that has until re-

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cently been considered only as a participant in oxidative phosphorylation in mitochondria [10]. However, seminal work of Liu et al demonstrated that cytoplasmic cytochrome c was responsible for activation of caspase-3 [11]. A current understanding of the signal transduction pathway suggests that upon release from mitochondria, cytochrome c binds the complex that contains apoptotic protease activating factor (Apaf-1) and procaspase-9 in the cytoplasm and thereby catalyzes enzymatic activation of caspase-9, which in turn activates caspase-3 to initiate apoptosis [12]. Perhaps the strongest controls of mitochondrial-induced apoptosis are the Bcl-2 family proteins. This family can be divided into those molecules that prevent apoptosis, such as Bcl-2 and Bcl-X_L [13, 14], and those that promote apoptosis, such as Bax, Bad, and Bcl-X_S [13, 15, 16]. While the mechanism of action of these proteins is open to debate [10], recent evidence suggests the Bcl-2 family, through a complex mechanism of homodimerization and heterodimerization among the family members, is directly involved in mitochondrial release of cytochrome c [15–21].

Apoptosis was recently demonstrated in kidneys of S rats exposed to 8.0% NaCl diet for three weeks. An increase in apoptosis in both glomerular and tubular compartments was observed [22]. These findings occurred at a time when renal function was markedly impaired and irreversible changes in renal morphology developed [5]. The present series of experiments was designed to determine whether apoptosis began in kidneys of hypertensive S rats before morphological and functional evidence of renal injury and further to characterize the role of cytochrome c in this process.

METHODS

Animal preparation

Studies were conducted using 24 male Dahl/Rapp salt-sensitive (SS/Jr, termed S) and 24 male SD rats, 28 days of age, obtained from Harlan Sprague-Dawley (Indianapolis, IN, USA). The rats were housed under standard conditions and given a formulated diet (AIN-76 A; Dyets, Inc., Bethlehem, PA, USA) that contained either 0.3 or 8.0% NaCl. These diets were prepared specifically to be identical in protein and electrolyte composition and differed only in NaCl and sucrose content. Groups of S and SD rats on 0.3 and 8.0% NaCl were maintained contemporaneously for each of the experiments performed. On days 7 and 21 of study, rats from all of the groups were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg; Abbott Laboratories, North Chicago, IL, USA). Laparotomy was performed, and the kidneys were perfused in situ through the aorta with cold isotonic heparinized saline until blanching occurred, which generally required infusion of 50 to 60 mL saline over two minutes. Both kidneys were harvested under sterile conditions to obtain protein for Western blotting and enzyme-linked immunosorbent assay (ELISA). Kidney tissue was also placed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for histochemistry, and some kidney tissue was also used to isolate mitochondria, as described later in this article.

In situ detection of DNA fragmentation using TUNEL

In situ detection of DNA fragmentation was performed simultaneously on tissue sections of S and SD rats on the two diets by incorporation of fluorescein-12-dUTP at the 3′-OH ends of DNA using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL assay; Apoptosis Detection System, Fluorescein; Promega, Madison, WI, USA). Paraffin-embedded sections (5 μm in thickness) were pretreated to deparaffinize the sections and permeabilize the tissue using proteinase K followed protocols provided by the manufacturer of the TUNEL assay kit. Following pre-equilibration in 100 μL of buffer containing 200 mmol/L potassium cacodylate, 25 mmol/L Tris-HCl, 0.2 mmol/L dithiothreitol (DTT), 0.25 mg/mL bovine serum albumin (BSA), and 2.5 mmol/L cobalt chloride, strands of DNA were end labeled by incubation at 37°C for one hour in 50 μmol/L fluorescein-12-dUTP, 100 μmol/L dATP, 10 mmol/L Tris-HCl (pH 7.6), 1 mmol/L ethylenediaminetetraacetic acid (EDTA) and terminal deoxynucleotidyl transferase; the reaction was stopped by immersing the slides in 2 × standard saline citrate (SSC; 1 × SSC is 15 mmol/L sodium citrate, pH 7.0, in 0.15 mol/L NaCl) for 15 minutes at room temperature. The slides were then stained by immersion in propidium iodide (Sigma Chemical Co., St. Louis, MO, USA), 1 μg/mL in PBS, for 15 minutes in the dark. After washing, the samples were mounted and examined and photographed at ×40 magnification using a fluorescence microscope (Leica, Heidelberg, Germany) equipped with a digital camera (Model C5810; Hamamatsu Photonics K.K., Hamamatsu, Japan). For fluorescein, the excitation and barrier filters were set at 450 to 490 and 515 to 560 nm, respectively. Red fluorescence of propidium iodide was observed using excitation and barrier filters of 515 to 560 and 580 nm, respectively.

Apoptosis quantitation by nucleosome detection

Cytoplasmic content of histone associated-DNA fragments (mononucleosomes and oligonucleosomes) in kidney cortex was quantitated using ELISA (Cell Death Detection ELISA^{Plus}; Boehringer Mannheim, Mannheim, Germany), following the protocol provided by the manufacturer. Briefly, cytoplasmic extracts of kidney cortex were obtained simultaneously from S and SD rats on the two diets using lysis buffer provided in the kit. Twenty microliters of each sample were transferred into strepta-

vidin-coated wells of a microplate. Eighty microliters of the immunoreagent mix, which contained biotin-labeled monoclonal antihistone antibody and peroxidase-conjugated monoclonal anti-DNA antibody in incubation buffer, were added to each well and incubated for two hours at room temperature with vigorous shaking (300 r.p.m.). The wells were washed three times with incubation buffer, and color was developed by adding 100 μL of peroxidase substrate solution into each well and incubating at room temperature with shaking (250 r.p.m.) about 10 minutes. Optical density was determined at 405 nm using a microplate reader ($THERMO_{max}$; Molecular Devices Corp., Menlo Park, CA, USA).

Isolation of mitochondria

Isolation of purified mitochondria was performed in standard fashion [23–26]. Renal cortical fragments from S and SD rats on the 0.3 and 0.8% NaCl diets for 7 and 21 days were submerged in ice-cold mitochondrial isolation buffer (MIB) containing 210 mmol/L mannitol, 70 mmol/L sucrose, 0.5 mmol/L EGTA, 5 mmol/L HEPES, pH 7.5, 0.45% BSA, and a mixture of protease inhibitors (Complete; Boehringer Mannheim). All subsequent steps were performed on ice or at 4°C. The tissue was chopped into 1 to 2 mm^3 cubes and homogenized by hand using a B-type pestle. Large cell debris and nuclei were pelleted by centrifuging twice for five minutes at 600 × *g*. The mitochondria were pelleted by centrifuging the supernatant for 10 minutes at 13,000 × *g*. The pellets were resuspended in 2 mL of MIB and layered on top of a discontinuous sucrose gradient formed by layering 12 mL of 1.2 mol/L sucrose, 10 mmol/L HEPES, pH 7.5, 1 mmol/L EDTA, 0.1% BSA on top of 15 mL of 1.6 mol/L sucrose, 10 mmol/L HEPES, pH 7.5, 1 mmol/L EDTA, 1% BSA. The samples were centrifuged at 27,000 r.p.m. for two hours in a Beckman SW28 rotor (Fullerton, CA, USA) at 4°C. Mitochondria, which were recovered at the interface between 1.6 and 1.2 mol/L sucrose, were washed and then resuspended in MIB containing 0.5 instead of 1 mmol/L EGTA.

Cytochrome c release assay

Mitochondria (100 μg total protein) isolated from each S and SD rat were incubated for 10, 30, and 60 minutes separately at 30°C in 200 μL KCl buffer (125 mmol/L KCl, 0.5 mmol/L EGTA, 5 mmol/L succinate, 10 mmol/L HEPES-KOH, pH 7.4, 4 mmol/L $MgCl_2$, 5 mmol/L Na_2HPO_4, 5 μmol/L rotenone). Mitochondria were then centrifuged at 13,000 × *g* for 10 minutes at 4°C. The pellet was solubilized in radioimmunoprecipitation assay (RIPA) buffer and analyzed by cytochrome c immunoassay. Supernatant fractions from these experiments were used either for analysis of cytochrome c release using immunoassay or to determine capability to activate caspase-3 in a cell-free apoptotic system described later in this article.

Cytochrome c immunoassay

Concentrations of cytochrome c were quantitated using ELISA (Quantikine Cytochrome c Immunoassay; R&D systems Inc. Minneapolis, MN, USA), following the protocol provided by manufacturer. This assay used a sensitive sandwich enzyme immunoassay technique. Briefly, 75 μL of antirat cytochrome c conjugated to horseradish peroxidase were added to microtiter wells coated with a monoclonal antibody specific for rat cytochrome c, and then 50 μL of sample were added. The solution was gently mixed for one minute and incubated for two hours at room temperature. The wells were washed five times with 400 μL of wash buffer. Color was developed in standard fashion, and optical density was determined at 450 nm using a microplate reader ($THERMO_{max}$; Molecular Devices Corp.).

Preparation of tissue lysates

Samples of renal cortex from S and SD rats on 0.3 and 8.0% NaCl diets for 7 and 21 days were diced into small pieces. Each gram of tissue was placed in 3 mL of ice-cold RIPA buffer, which contained a combination of protease inhibitors (Complete; Boehringer Mannheim), and then homogenized (Omni-Mixer 17105; Omni, Waterbury, CT, USA) in a standard fashion. The samples were then centrifuged at 10,000 × g for 20 minutes at 4°C. The supernatant represented total cell lysate. In addition, cytoplasmic extracts were also prepared from renal cortices. The tissue was washed with ice-cold PBS and chilled in a hypotonic extraction buffer (HEB) with the protease inhibitor cocktail (Complete). The tissue was then disrupted using 50 strokes in a prechilled Dounce homogenizer with a tight-fitting pestle. The samples were sedimented at 800 × *g* for 10 minutes. The postnuclear supernatant was obtained by centrifugation at 100,000 × *g* for 60 minutes in a Beckman TL-100 Ultracentrifuge at 4°C. The resulting cytosolic extract did not contain whole cells, nuclei, and mitochondria. Total protein of each sample was determined using a kit (Micro BCA protein assay reagent kit; Pierce, Rockford, IL, USA).

Western blot analysis

Western blot analysis was performed in a standard fashion [27–30]. Electrophoresis of proteins was performed using either 8 or 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Equal amounts of total protein (5 to 60 μg) were boiled in SDS-Laemmli sample buffer for five minutes and then loaded into each lane. The proteins were separated under reducing conditions at 80 V. Western blot transfer of separated protein was performed at 4°C using nitrocellulose membranes at 200

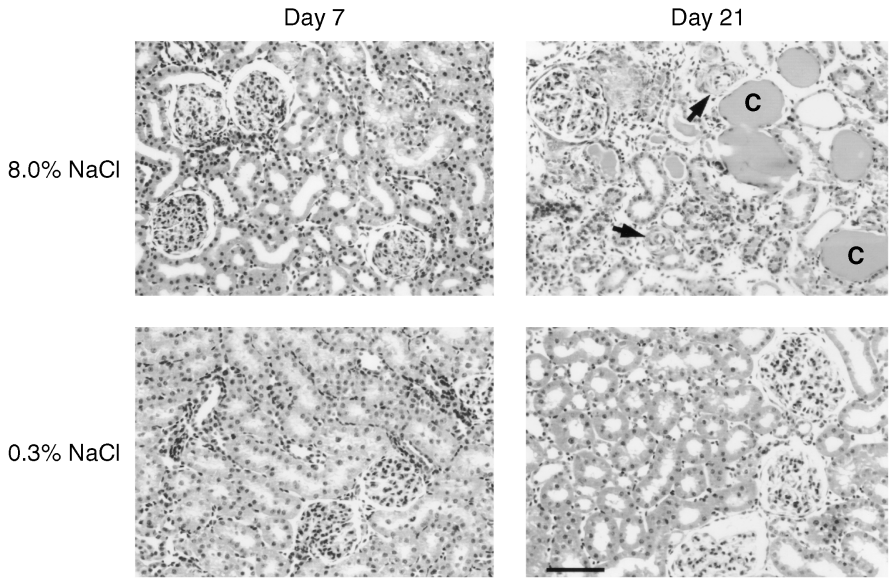

Fig. 1. Representative light micrographs of kidneys from Dahl/Rapp salt-sensitive (S) rats on 0.3 and 8.0% NaCl for 7 and 21 days. On day 7, renal morphology was not altered. By day 21, damage to the tubulointerstitium was apparent. Tubular atrophy, dilation, and intraluminal cast formation were seen. The interstitium was expanded, as indicated by separation of the tubule segments in the specimen. Thickening of the arterioles was evident (arrows). (HE stain; black bar represents 100 μm. C, intratubular cast.)

mA for two hours. Membranes were incubated for two hours in Tris-buffered saline (TBS)-Tween (10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, and 0.1% Tween) containing 10% nonfat dry milk. The membranes were probed with an appropriate dilution (1:500 to 1:2000) of primary antibody in TBS-Tween containing 5% nonfat dried milk for two hours. Anti-cytochrome c rabbit polyclonal antibody, anticaspase-3 p20 goat polyclonal antibody, and anticaspase-9 p10 rabbit polyclonal antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit polyclonal antibody raised against poly(ADP-ribose) polymerase (PARP) p85 fragment was purchased from Promega Corp. The blots were washed for one hour with frequent changes of TBS-Tween and then incubated with the appropriate horseradish peroxidase-conjugated anti-IgG antibody (Bio-Rad Inc., Hercules, CA, USA), 1:20,000 dilution, in TBS-Tween containing 5% nonfat dried milk. After five additional washes using TBS-Tween, membranes were developed using the ECL Western blotting system and Hyperfilm (Amersham International plc., Buckinghamshire, UK). The films were scanned using a densitometer (Model 620 Video Densitometer; Bio-Rad Inc.) to quantitate bands.

Preparation and induction of a cell-free apoptotic system

Preparation of a cell-free apoptotic assay system was performed as described [24, 26, 31]. HeLa cells (CCL 2.2; American Type Culture Collection, Manassas, VA, USA) were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), in an atmosphere of 5% CO. At 80% confluence, the flasks were placed on ice and washed twice with ice-cold PBS, and cells were harvested using a cell scraper. Cells were then washed and suspended in one pellet volume of ice-cold HEB, which contained 20 mmol/L HEPES (pH 7.5), 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L DTT, and 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF). Cells were incubated for 20 minutes on ice and then disrupted using 30 strokes using a B-type pestle and a prechilled Dounce homogenizer. Cell lysates were centrifuged at 16,000 × *g* for 30 minutes. The clarified supernatant was used immediately or stored in aliquots at −80°C. The cytoplasmic fraction did not contain microscopically visible whole cells or cell fragments.

Determination of caspase-3 enzymatic activity

HeLa cell cytoplasmic extract was prepared as described previously in this article. Purified mitochondria (500 μg protein in 50 μL MIB) were incubated in 20 μL of cytosolic extract (100 μg total protein) and 1 mmol/L dATP for one hour at 30°C. Mitochondria were then removed by centrifugation, and the resulting supernatant was analyzed for caspase-3 activity using a kit (Caspase-3 Colorimetric Assay Kit; R&D Systems, Inc.). Following the protocol provided by the manufacturer, after incubation in the cell-free condition as described previously in this article, the ability of the cytosolic preparations to cleave DEVD p-nitroanilide (p-NA) was determined. Briefly, reactions were assembled by adding 50 μL of cytosol, 50 μL of 2 × reaction buffer and 5 μL of caspase-3 colorimetric substrate (DEVD-pNA) to wells of a microtiter plate. Plates were incubated at 37°C and release of free pNA, which absorbs at 405 nm, was monitored ($THERMO_{MAX}$; Molecular Devices Corp.) con-

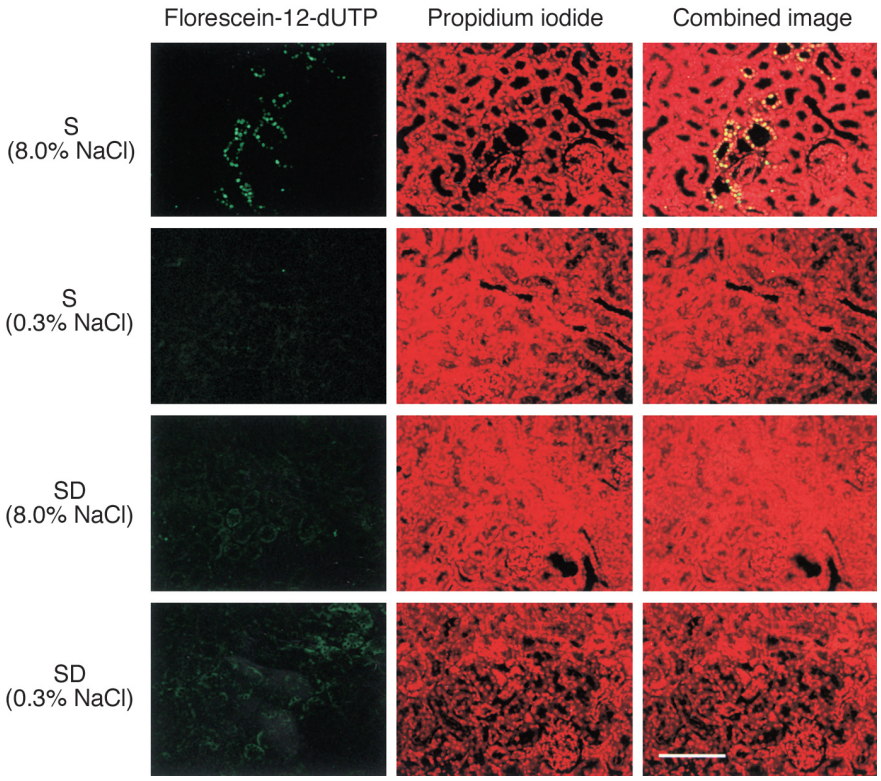

Fig. 2. Representative TUNEL stains of kidneys from Dahl/Rapp salt-sensitive (S) and Sprague-Dawley (SD) rats on 0.3 and 8.0% NaCl diets for seven days. The panels on the left are fluorescein-labeled cells from TUNEL. The middle panels show cells that stain with propidium iodide, and the panels on the right show a composite of the two labels. Dual-labeled cells appear yellow (white bar = 100 μm).

tinuously for two hours. In related experiments, 50 μL samples of supernatant from the cytochrome c release assay (described previously in this article) were also assayed for caspase-3 activity.

In other experiments, caspase-3 activity was determined in renal cortical lysates (200 μg total protein) obtained from S and SD rats on the two diets for 7 and 21 days, using this same assay.

Statistical analysis

All data were presented as mean ± SE. Significant differences were determined using either the unpaired *t* test or one-way analysis of variance with standard post hoc testing (Statview, version 4.5; Abacus Concepts, Inc., Berkeley, CA, USA), where appropriate. A *P* value of <0.05 assigned statistical significance.

RESULTS

Hypertensive renal damage was severe by day 21 of study

Over the course of the study, S and SD rats tolerated both diets well. However, previous studies showed that S rats rapidly developed hypertension on the 8.0% NaCl diet, and blood pressure continued to increase over 21 days on the 8.0% NaCl diet [5, 22]. In addition, inulin

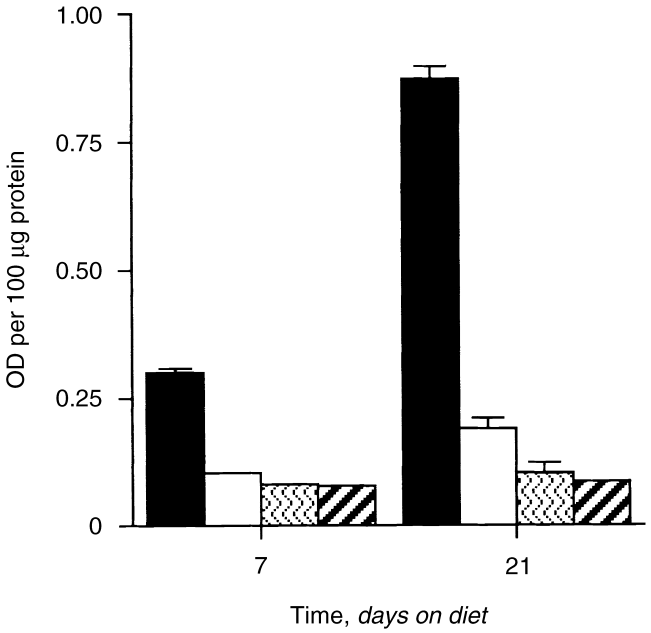

Fig. 3. Quantitation of cytoplasmic nucleosome content demonstrates significant increases ($P < 0.05$) in samples of kidney cortex from S rats on the 8.0% NaCl diet compared with the other three groups (N = 4 rats in each group). The increase was seen by day 7 and appeared to increase over the course of study. Symbols are: (■) S 8.0% NaCl diet; (□) S 0.3% NaCl diet; (▨) SD 8.0% NaCl diet; (▨) SD 0.3% NaCl diet.

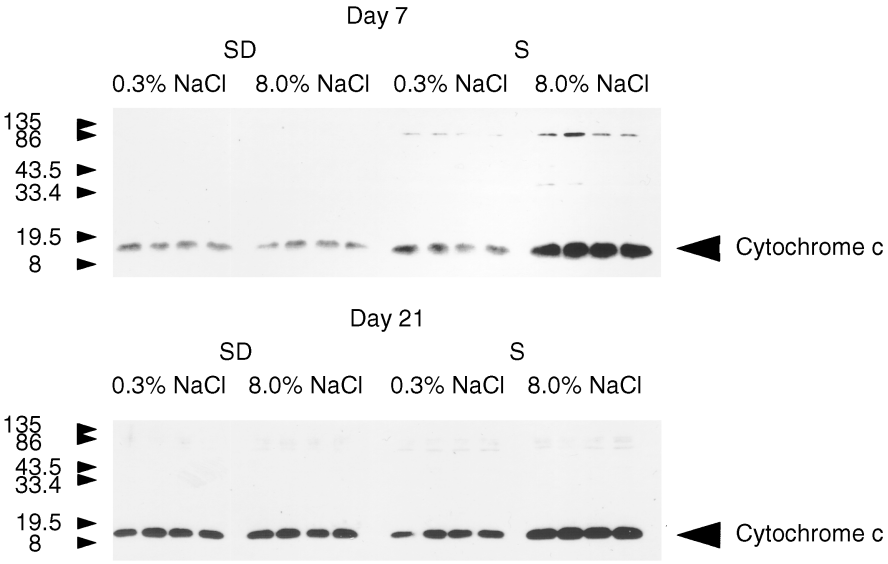

Fig. 4. Using a rabbit polyclonal antibody to cytochrome c, Western blot demonstrates an increase in cytoplasmic content of cytochrome c specifically in kidneys of S rats exposed to the high-salt diet ($N = 4$ rats in each group).

clearance and albumin excretion rates remained normal at day 7 but indicated substantial damage by day 21 [5, 22]. Histologic examination of kidneys from both S and SD rats on both diets was performed on days 7 and 21 (Fig. 1). At day 7, light microscopic examination of kidneys of S rats on either diet demonstrated relatively normal morphology with preservation of the tubulointerstitium. By day 21, kidneys of S rats on 8.0% NaCl diet showed severe injury with expansion of the mesangium, tubular atrophy and tubular cell loss, intraluminal cast formation, and expansion of the interstitium, as demonstrated by the increased distance between the tubules. By light microscopy, renal morphology of SD rats on either diet did not show any abnormality.

Apoptosis was evident by day 7 of study

Using paraffin-embedded tissue, examples of TUNEL-positive cells were observed in tubules and, to a lesser extent, in glomeruli from all S rats on 8.0% NaCl diet for seven days (Fig. 2). By day 21, TUNEL-positive cells were widespread in tubules and glomeruli. In each section, the number of TUNEL-positive cells varied and was severe in some sections, particularly in rats on the diet for three weeks. On days 7 and 21, scattered TUNEL-positive cells were also seen in kidneys from SD rats on both diets and in S rats on 0.3% NaCl diet. Because endonucleases do not have access to DNA wound around histones and instead cleave DNA that spans between the histones, the apoptotic process typically promotes release of nucleosomes into the cytoplasm. Cytoplasmic nucleosome content was quantitated on days 7 and 21 and was increased only in kidney cortex from S rats on 8.0% NaCl (Fig. 3).

Mitochondria from kidneys of S rats on 8.0% NaCl diet released cytochrome c

Cytoplasmic cytochrome c content was determined using Western blotting (Fig. 4). Mean optical density of cytochrome c from cytoplasmic extracts of kidneys of S rats on the high-salt diet for seven days was 1.82 ± 0.17, which was greater ($P < 0.05$) than the mean densities of cytochrome c from S rats on the 0.3% NaCl diet (0.87 ± 0.13), SD rats on the 8.0% NaCl diet (0.78 ± 0.1), and SD rats on the 0.3% NaCl diet (0.86 ± 0.04). Similarly, the mean optical density of cytochrome c from kidneys of S rats on the 8.0% NaCl diet for 21 days was 2.17 ± 0.22, which was greater ($P < 0.05$) than densities of cytochrome c in corresponding groups of S rats on the 0.3% NaCl diet (0.70 ± 0.13) and SD rats on the 8.0% (0.76 ± 0.08) and 0.3% (0.73 ± 0.20) NaCl diets. Incubation of isolated mitochondria from S rats on the 8.0% NaCl diet showed release of cytochrome c into the supernatant; associated with this release was a decrease in the mitochondrial content of cytochrome c (Fig. 5). These findings did not occur with mitochondrial preparations that were harvested simultaneously from the other groups of rats. Using a cell-free system prepared from HeLa cytosol, only mitochondria isolated from S rats on a 8.0% NaCl diet activated caspase-3, as determined by cleavage of DEVD-pNA, a caspase-3 substrate (Fig. 6). Thus, mitochondria alone were sufficient to activate the apoptotic process.

Caspase-9 and caspase-3 were activated in kidney cortex from S rats on 8.0% NaCl for seven days

Release of cytochrome c into the cytoplasm has been shown to promote cleavage of pro-caspase-9 and subsequent activation of caspase-3 [12]. Western blots of ly-

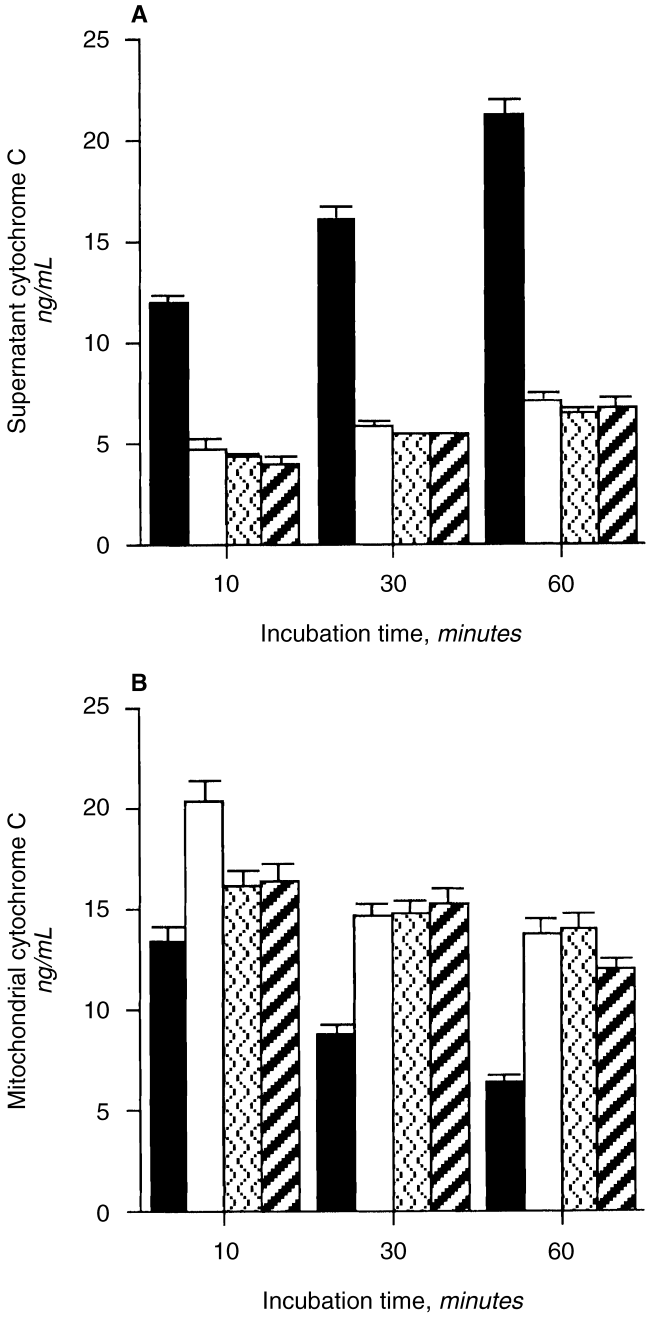

Fig. 5. Incubation of mitochondria isolated from kidney cortex of S rats on the high-salt diet for seven days showed an abnormally increased ($P < 0.05$) release of cytochrome c into the supernatant (*A*) and loss ($P < 0.05$) of cytochrome c from mitochondria over time (*B*) compared with the other three groups of rats ($N = 4$ rats in each group). Symbols are: (■) S 8.0% NaCl diet; (□) S 0.3% NaCl diet; (▨) SD 8.0% NaCl diet; (▨) SD 0.3% NaCl diet.

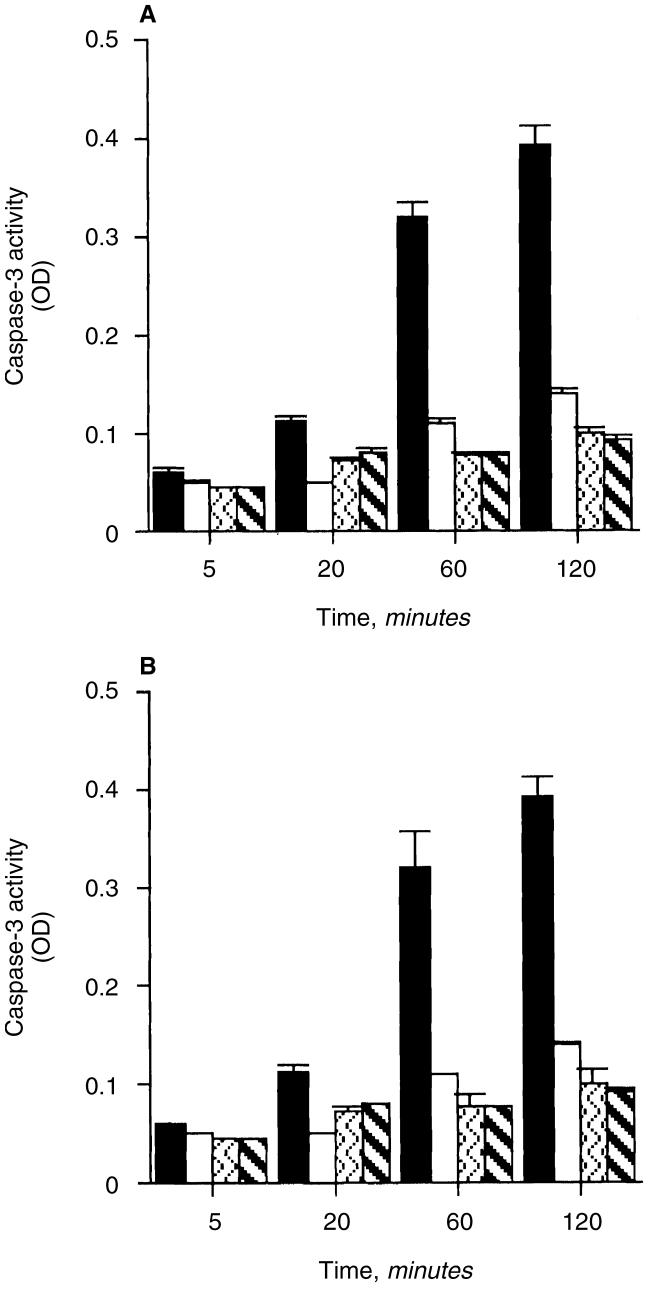

Fig. 6. Mitochondria isolated from kidney cortex at day 7 were incubated in a cell-free system that contained a specific caspase-3 substrate (DEVD-pNA). Cleavage of this substrate releases pNA, which is then detected at 405 nm. Mitochondria from S rats on the high-salt diet demonstrated increased ($P < 0.05$) capability of activating caspase-3, compared with the other three groups ($N = 4$ rats in each group, *A*). Experiments that replaced the cytosol with buffer alone did not demonstrate caspase-3 activity (data not shown). Aliquots of supernatant obtained from incubation of isolated mitochondria were also used in the cell-free system (*B*). Compared with the other three groups, the supernatant fraction from mitochondria of S rats on the high-salt diet demonstrated increased ($P < 0.05$) activation of caspase-3 ($N = 4$ rats in each group). Thus, the combined data showed that mitochondria from these samples were sufficient alone to activate the apoptotic cascade. Symbols are: (■) S 8.0% NaCl diet; (□) S 0.3% NaCl diet; (▨) SD 8.0% NaCl diet; (▨) SD 0.3% NaCl diet.

sates from kidney cortex demonstrated the presence of active caspase-9 only in kidneys from S rats on the 8.0% NaCl diet (Fig. 7). The active form of caspase-3 was also identified specifically in these kidneys (Fig. 8). Using the caspase-3 colorimetric substrate (DEVD-pNA), lysates from kidney cortex of S rats on the 8.0% NaCl diet also

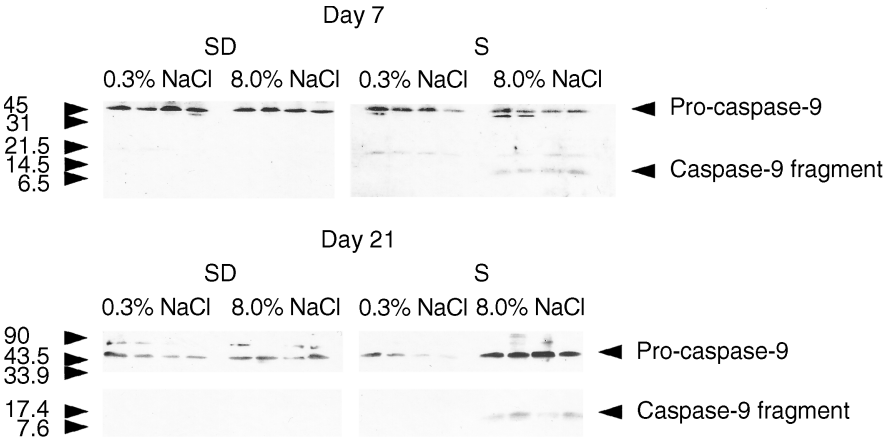

Fig. 7. Using a rabbit polyclonal antibody that recognizes the carboxyl terminal portion of caspase-9, Western blot analysis of lysates of kidney cortex showed the presence of the caspase-9 fragment, indicating activation of caspase-9, specifically in S rats on the high-salt diet (N = 4 rats in each group).

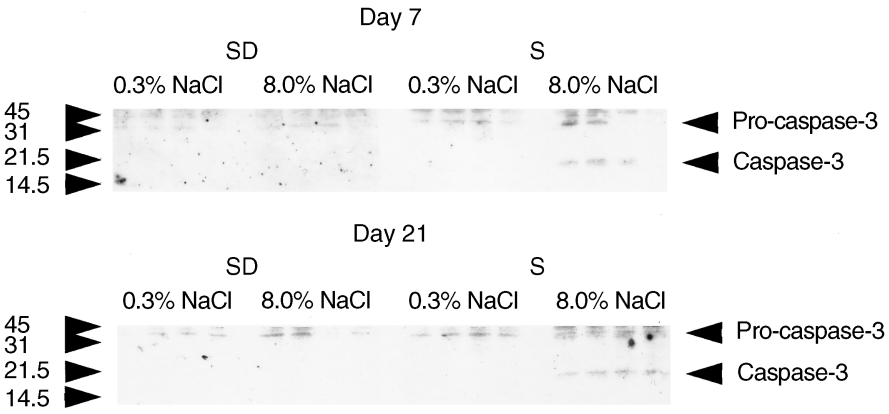

Fig. 8. Cleavage of procaspase-3 to produce the active form of the enzyme was shown by Western blotting using a goat polyclonal antibody directed against the carboxyl terminal portion of caspase-3. Only lysates from kidney cortex of S rats on the high-salt diet contained active (20 kD) caspase-3 (N = 4 rats in each group).

showed functional caspase-3 activity (Fig. 9). Finally, one of the early nuclear targets of caspase-3 is PARP [32–34]. Caspase-3 cleaves PARP to produce an inactive approximately 85 kD fragment. Using a polyclonal antibody that specifically recognizes the p85 fragment of PARP, but does not recognize the intact 116 kD form of the molecule, PARP cleavage was observed in kidneys of S rats exposed to the high-salt diet (Fig. 10).

DISCUSSION

The Dahl/Rapp S rat is a genetic model of low-renin, salt-sensitive hypertension [3], but these animals also develop renal failure in a rapid and reproducible manner and have pathological renal changes that are very similar to the fibrotic process in humans known as hypertensive nephrosclerosis [5]. Thus, this model may be useful in understanding hypertensive nephrosclerosis that occurs particularly in defined populations of humans with low-renin essential hypertension. Renal failure in this model becomes clinically manifest at two weeks with appearance of albuminuria and depressed inulin clearance with the animal on 8.0% NaCl diet. Reversing the hypertension at this time point results in the return of inulin clearance and renal morphology to normal. By three weeks on the same diet, inulin clearance is severely depressed. Renal morphological abnormalities include glomerulosclerosis, arteriolosclerosis, and interstitial scarring and tubular cell dropout [5]. In the present study, these same changes in renal morphology were again seen; renal morphology was well preserved in S rats on the 8.0% NaCl for seven days (Fig. 1). By day 7, however, TUNEL-positive cells were seen in kidneys of these rats. Apoptosis was confirmed by demonstration of elevated levels of cytoplasmic nucleosomes, activation of caspase-3, and cleavage of PARP. The increase in apoptosis occurred prior to the demonstration of abnormalities in renal morphology and inulin clearance, and persisted through day 21 when renal morphology was grossly abnormal and inulin clearance was severely depressed [5]. Thus, acceleration of apoptosis preceded functional deterioration in inulin clearance and onset of glomerulosclerosis and tubulointerstitial injury and fibrosis. Cytoplasmic content of cytochrome c was elevated in these kidneys and mitochondrial isolation studies confirmed that mitochondria from kidneys of S rats on the 8.0%

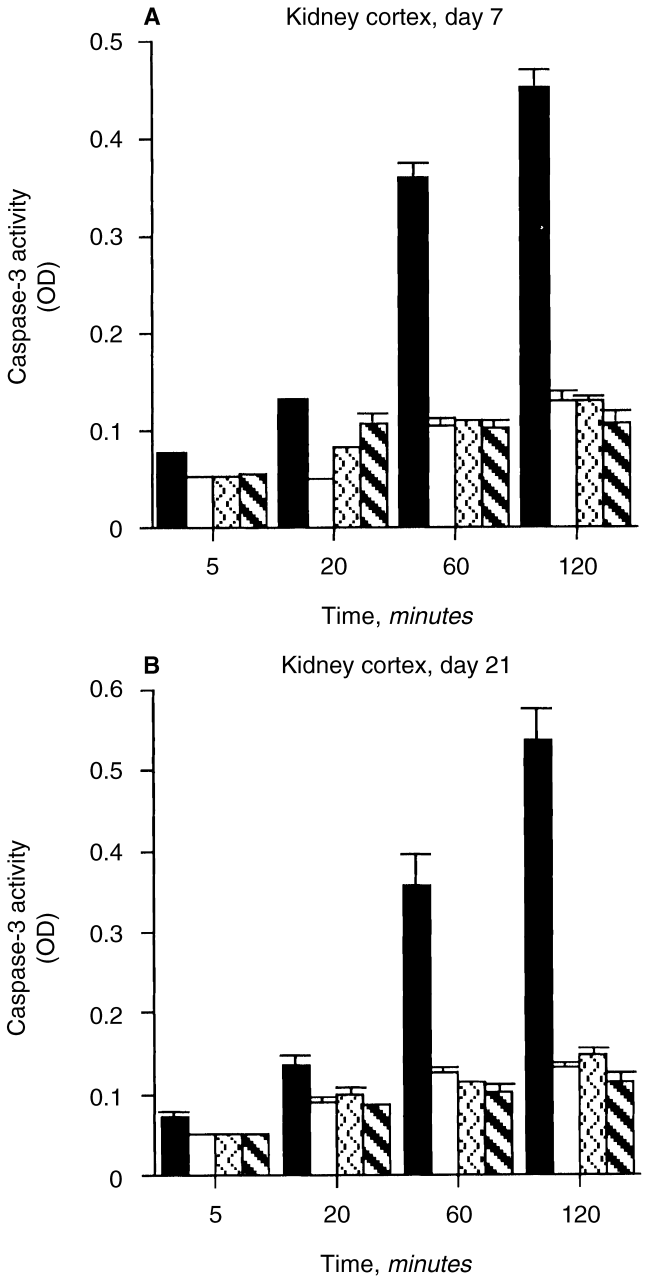

Fig. 9. To demonstrate functional activation of caspase-3, lysates of kidney cortex were incubated with the caspase-3 substrate (DEVD-pNA), and release of pNA was monitored. At each day of study, lysates from S rats on the high-salt diet showed increased ($P < 0.05$) caspase-3 function, compared with the other three groups ($N = 4$ rats in each group). Symbols are: (■) S 8.0% NaCl diet; (□) S 0.3% NaCl diet; (▨) SD 8.0% NaCl diet; (▨) SD 0.3% NaCl diet.

NaCl diet released cytochrome c. The addition of these mitochondria to a cell-free system activated caspase-3 in vitro, confirming that mitochondria alone from these animals were sufficient to initiate the apoptotic cascade. Finally, in vivo activation of caspase-9 and caspase-3 were shown specifically in kidney cortex from S rats on the high-salt diet. The combined data showed that abnormal release of cytochrome c by mitochondria from kidneys of S rats on the high-salt diet was the trigger of the apoptotic mechanism.

The Bcl-2 family of proteins has been considered important determinants of the release of cytochrome c by mitochondria [15–21]. Previous work demonstrated increased renal cortical expression of pro-apoptotic molecules that included Bax and Bcl-X_S by day 21 on the high-salt diet. Bcl-X_S represents an alternative splice product of *bcl-x* and antagonizes the anti-apoptotic effect of Bcl-X_L [16]. Notably, expression of these molecules was not altered at day 7 [22]. Thus, while these pro-apoptotic members of the Bcl-2 family appeared to participate late in the course of renal failure, they did not appear to be solely responsible for the findings in the present study, because increased cytochrome c release was demonstrated to occur by day 7. Anti-apoptotic Bcl-2 family members, including Bcl-2 and Bcl-X_L, were also examined. Bcl-2 expression was not altered over the course of study, while Bcl-X_L was increased not only in the cortex from S rats but also SD rats on 8.0% NaCl diet. Despite an increase in Bcl-X_L, cytochrome c was released into the cytoplasm. Thus, at present, the Bcl-2 family proteins that were examined were not involved early in the course of this process. Up-regulation of Fas can also facilitate mitochondrial cytochrome c release by protease activation [35] and Fas was increased in kidneys of S rats exposed to the high-salt diet for 21 days, but was not observed at day 7; therefore, the Fas pathway did not appear to be responsible for the early phase of apoptosis in this model. The underlying initiating mechanism by which hypertension produced a mitochondrial release of cytochrome c in the kidney cortex of S rats has not yet been clarified.

In summary, these experiments demonstrated an early and persistent abnormal expression of apoptosis in kidneys of hypertensive S rats. Evidence of apoptosis preceded functional deterioration in inulin clearance and onset of glomerulosclerosis, tubulointerstitial injury and fibrosis, and contributed to the loss of renal function over time. Increased mitochondrial release of cytochrome c activated the apoptotic pathway that consisted of caspase-9 and subsequently caspase-3. Damage to the kidney almost certainly exacerbated hypertension, and blood pressure of S rats has been shown to increase over the three weeks of study [5, 22]. Because hypertension directly promotes renal injury in this model, a positive feedback loop was created. Understanding apoptosis in this model may improve the management of patients with end-organ kidney damage from hypertension.

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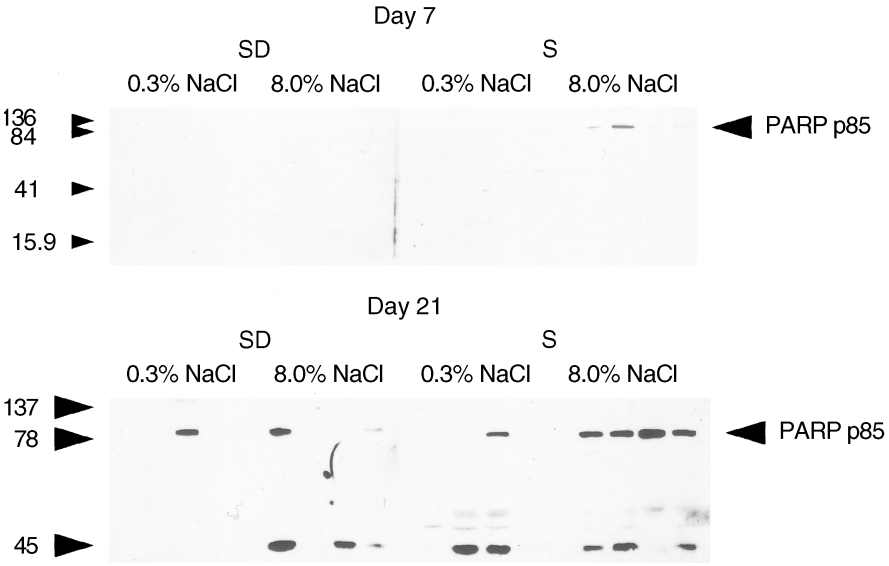

Fig. 10. Cleavage of poly(ADP-ribose) polymerase (PARP) to produce an inactive approximately 85 kD fragment represents an early nuclear event in the apoptotic cascade. Using a rabbit polyclonal antibody that recognizes specifically the 85 kD fragment but not intact 116 kD PARP, Western blot analysis of lysates of kidney cortex demonstrated the 85 kD fragment in S rats on the high-salt diet ($N = 4$ rats in each group) at days 7 and 21. While appearance of the p85 fragment was observed sporadically in the other samples, p85 PARP was identified in all samples of kidneys from S rats on the high-salt diet on both days of study.

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APPENDIX

Abbreviations used in this article are: BSA, bovine serum albumin; DTT, dithiothreitol; EDTA; ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; HEB, hypotonic extraction buffer; MIB, mitochondrial isolation buffer; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; p-NA, p-nitroanilide; RIPA, radioimmunoprecipitation assay; S, Dahl/Rapp salt-sensitive rat; SD, Sprague-Dawley rat; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; TBS, Tris-buffered saline; and TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

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